Supporting Online Material for

mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin

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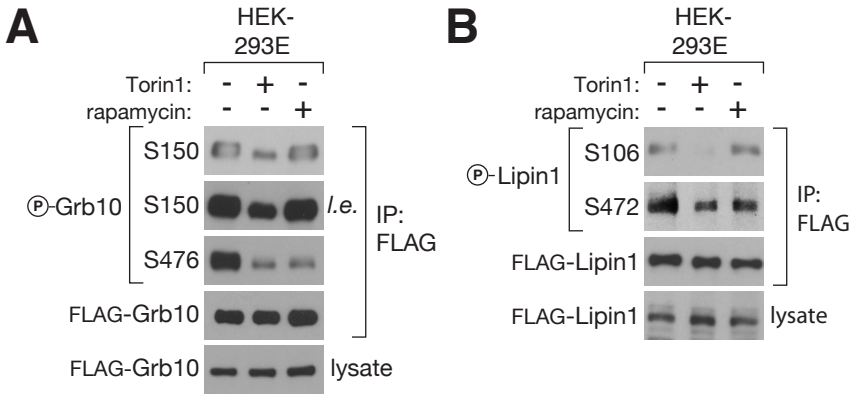
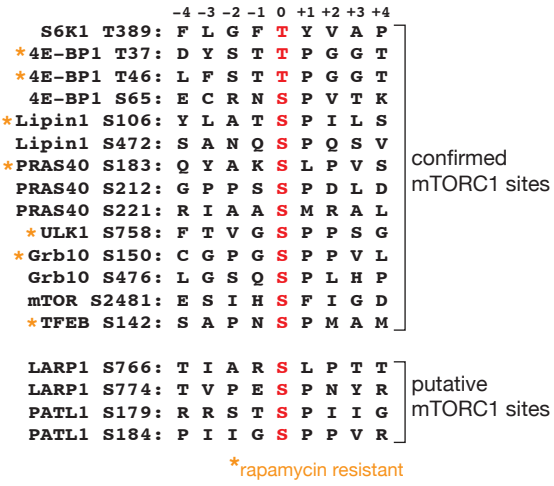
Supplemental Figure 1



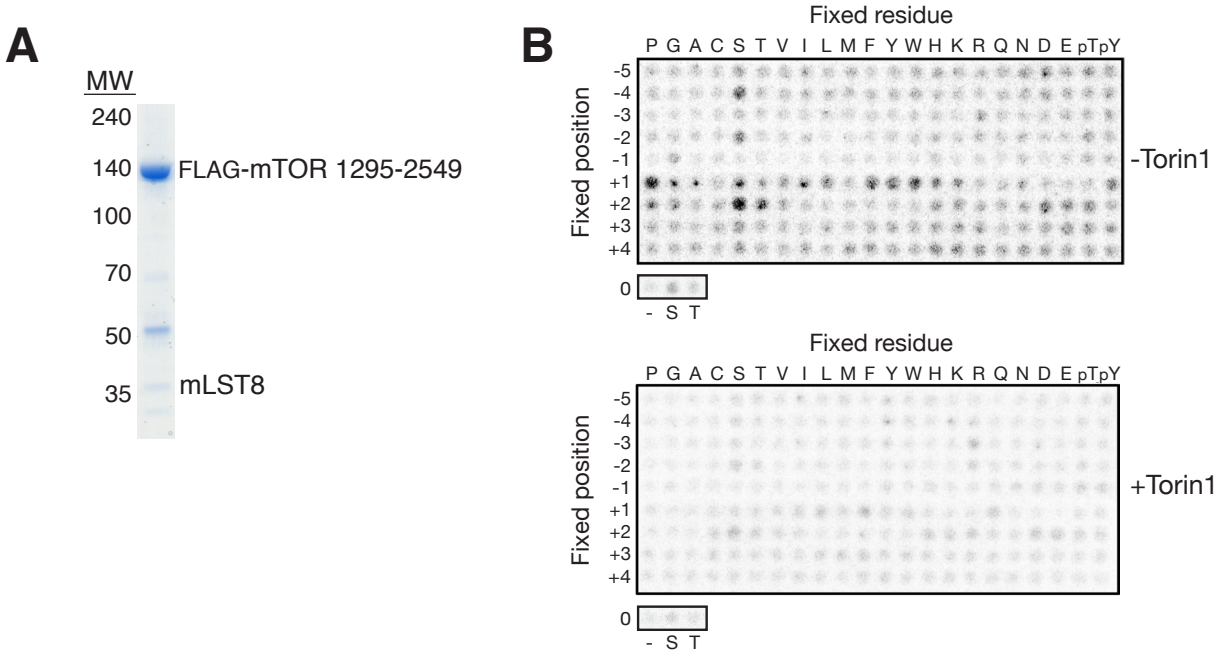
Supplemental Figure 1. HEK-293E cells were transfected with **(A)** FLAG-Grb10 and **(B)** FLAG-Lipin1 expression plasmids and treated with 100 nM rapamycin, 250 nM Torin1 or vehicle for 1 hr. Cell lysates and immunoprecipitates were analyzed by immunoblotting for the levels and phosphorylation states of Grb10 and Lipin1 (*l.e. = long exposure*).

Supplemental Figure 2



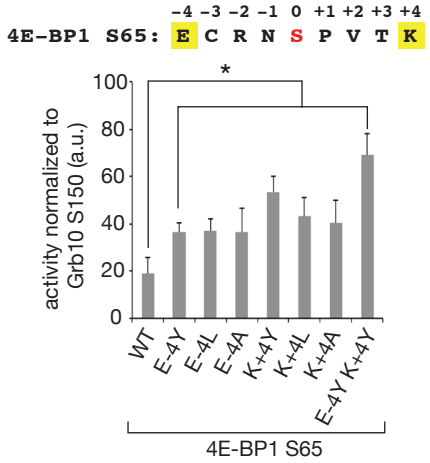
Supplemental Figure 2. Sequence alignment of known and putative mTORC1 phosphorylation sites from Figure 2B. Positions are numbered relative to the central phosphoacceptor serine or threonine and known rapamycin-resistant sites are indicated.

Supplemental Figure 3



Supplemental Figure 3. (A) Truncated mTOR mutant was purified from HEK-293T cells stably expressing FLAG-mTOR (1295-2549) coexpressed with mLST8. The purified complex was analyzed by SDS-PAGE followed by Coomassie staining. **(B)** *In vitro* phosphorylation of a PSPL with the truncated mTOR kinase domain in the presence and absence of 250 μM Torin1. Each reaction consists of a mixture of biotinylated peptides containing one fixed residue relative to the central phosphoacceptor and other residues randomized. In addition to 198 (9 × 22) peptide mixtures, two control peptide mixtures (bottom panel) bearing either Ser or Thr alone as the fixed phosphoacceptor residue in the context of a fully degenerate sequence are also included. These control mixtures serve as indicators of any preference the kinase has for either Ser or Thr residues at the phosphoacceptor site. Aliquots of each reaction were spotted onto a streptavidin membrane and analyzed by phosphoimaging. The numbering of the positions is relative to the central phosphoacceptor serine or threonine.

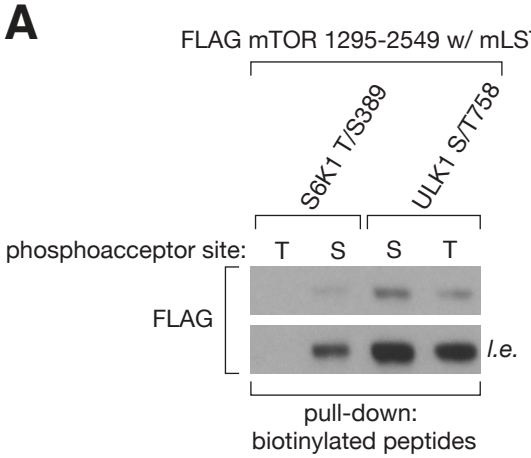
Supplemental Figure 4



Supplemental Figure 4. *In vitro* kinase activity of mTORC1 towards peptides containing indicated modifications to the 4E-BP1 S65 site were analyzed by autoradiography. Phosphorylation levels of the specified peptides were quantified by densitometry. Data are means ± S.D. (n = 3 to 5). (*P < 0.05) Note: Phosphoacceptor site is in red text and modified residues in yellow highlight.

Supplemental Figure 5

A

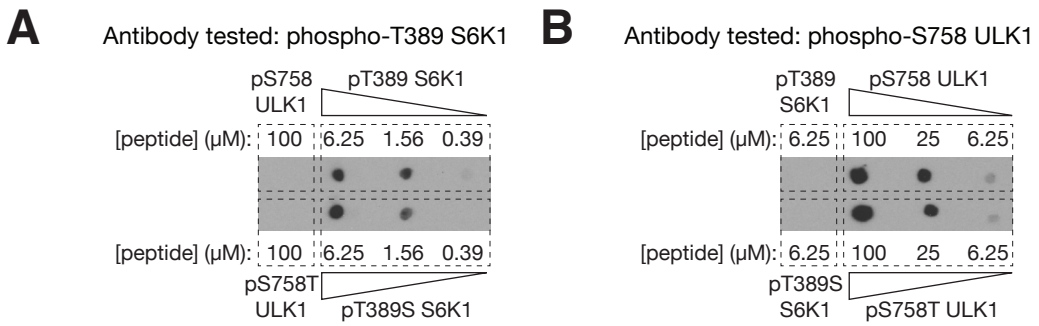


B

peptide	K_m (μM)	k_{cat} (min^{-1})	k_{cat} / K_m ($\mu M^{-1}\ min^{-1}$)
S6K1 T389	>> 1000*	-	-
S6K1 T389S	39.6 ± 12.6	1.4 ± 0.2	0.04 ± 0.01
ULK1 S758	6.3 ± 1.0**	18.0 ± 0.6**	2.85 ± 0.44
ULK1 S758T	24.1 ± 0.7**	21.4 ± 0.2**	0.88 ± 0.03
ULK1 S758 T-3A S+3A	26.2 ± 2.1	4.1 ± 0.3	0.15 ± 0.02
ULK1 S758T T-3A S+3A	56.8 ± 9.8	3.4 ± 0.1	0.06 ± 0.01

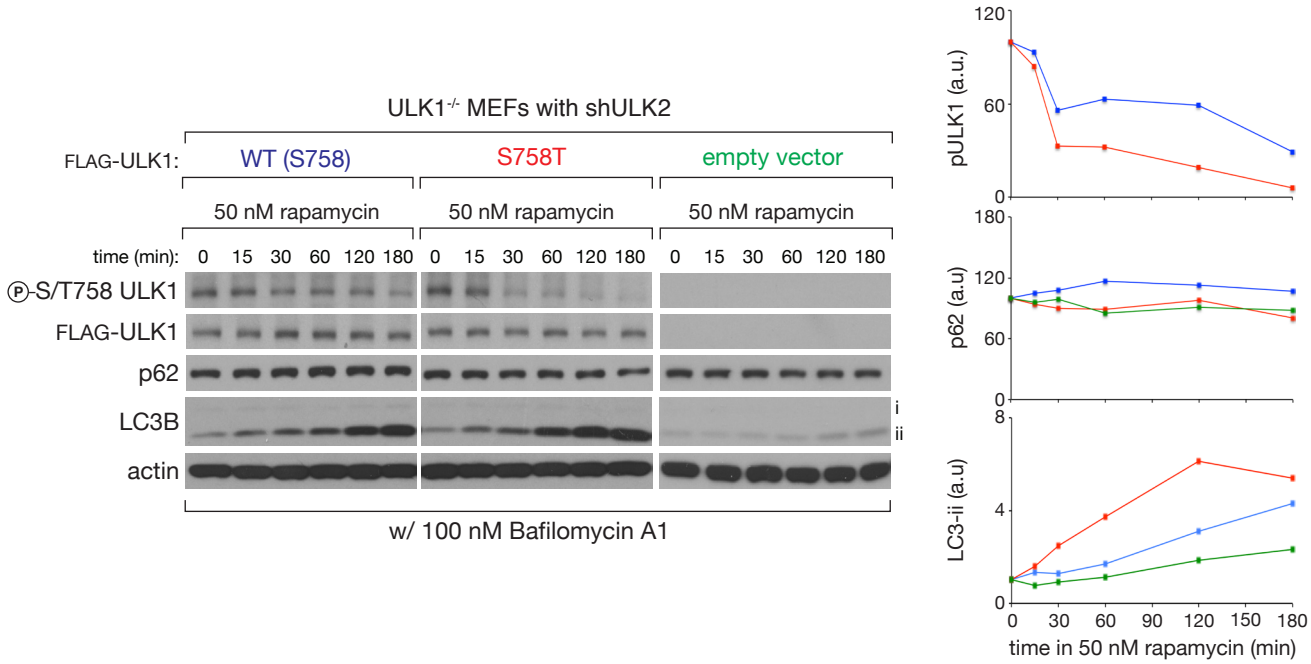
Supplemental Figure 5. (A) Binding of the S6K1 T389 and ULK1 S758 peptides containing either Ser or Thr at their phosphoacceptor sites to the mTOR kinase domain. A pull-down assay using streptavidin agarose was performed from a mixture of a biotinylated peptide encompassing an established mTORC1 phosphorylation site and the FLAG-tagged mTOR truncation mutant and analyzed by immunoblotting for the FLAG tag (*l.e. = long exposure*). **(B)** Steady-state kinetic measurements for individual peptides encompassing S6K1 T389, ULK1 S758 and S758 T-3A S+3A and their phosphoacceptor mutant counterparts, S6K1 T389S, ULK1 S758T and S758T T-3A S+3A. K_m and k_{cat} values of mTORC1 activity towards indicated peptide substrates were determined by measuring the rate of mTORC1 phosphorylation over a range of substrate peptide concentrations (0, 10, 100, 250, 500 and 1000 μM) at a non-limiting ATP concentration, 500 μM. The steady-state kinetic parameters were obtained by fitting the reaction rates to the Michaelis-Menten equation. *Note: K_m exceeds the highest concentration tested for the S6K1 T389 peptide. **Note: The better K_m and k_{cat} values for the ULK1 S758 and S758T peptides relative to other peptides may reflect the fact that the peptides contain more than one site phosphorylated by mTORC1 as demonstrated by kinetic parameters of mTORC1 activity towards the ULK1 S758 T-3A S+3A and ULK1 S758T T-3A S+3A peptides. See also Figure 3G.

Supplemental Figure 6



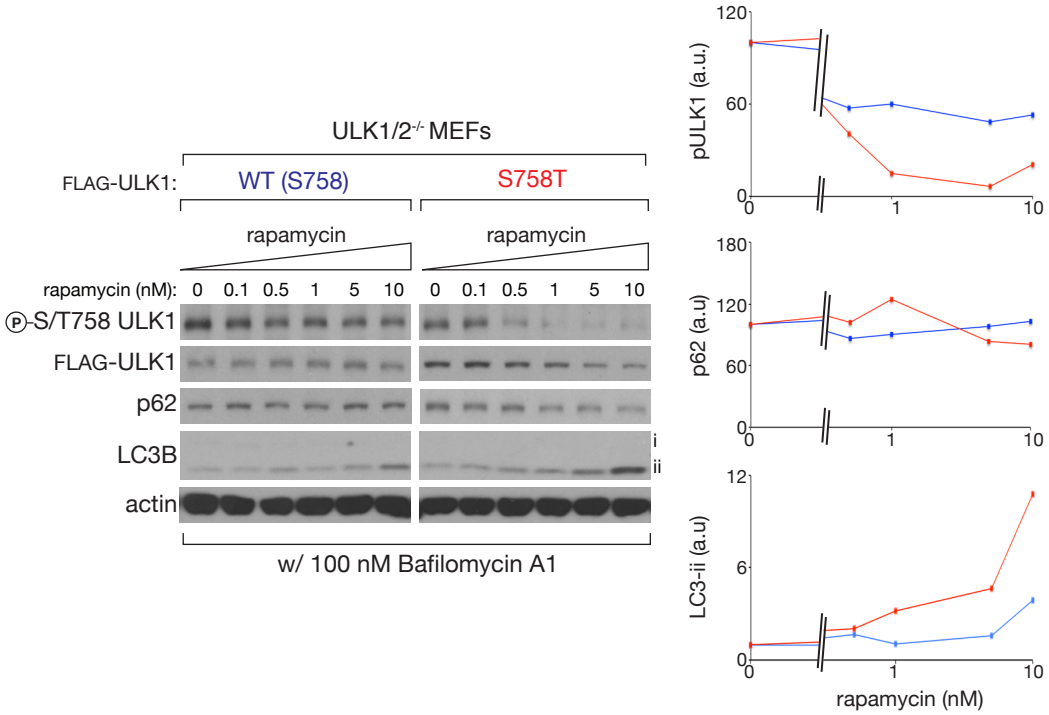
Supplemental Figure 6. Phosphospecific **(A)** T389 S6K1 and **(B)** S758 ULK1 antibodies from Cell Signaling Technology (#9206 and #6888, respectively) recognize phosphopeptides containing either Ser or Thr at their phosphoacceptor sites. 1 μL of biotinylated phosphopeptides at the indicated concentrations were spotted on a streptavidin coated membrane and analyzed by immunoblotting for the phosphorylation states of the specified peptides. Controls were S758 and S758T ULK1 peptides for (A) and T389 and T389S S758T peptides for (B).

Supplemental Figure 7



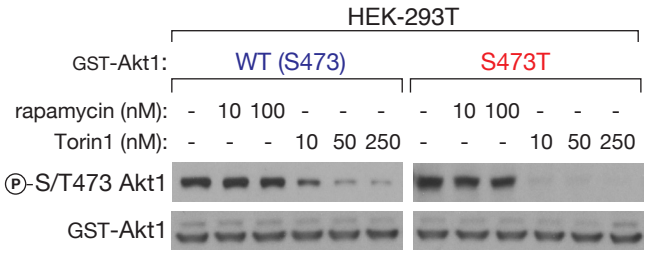
Supplemental Figure 7. Time-dependent responses of wild-type and mutant S758T ULK1 to rapamycin. ULK1$^{-/-}$ MEFs stably expressing an shRNA against ULK2 as well as FLAG-tagged wild-type ULK1, S758T ULK1 or an empty vector control were treated with 50 nM rapamycin in the presence of 100 nM Bafilomycin A1. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. Levels of pULK1, p62 and LC3-ii were quantified by densitometry (graphs) and normalized to levels of FLAG-ULK1 (actin for empty vector).

Supplemental Figure 8



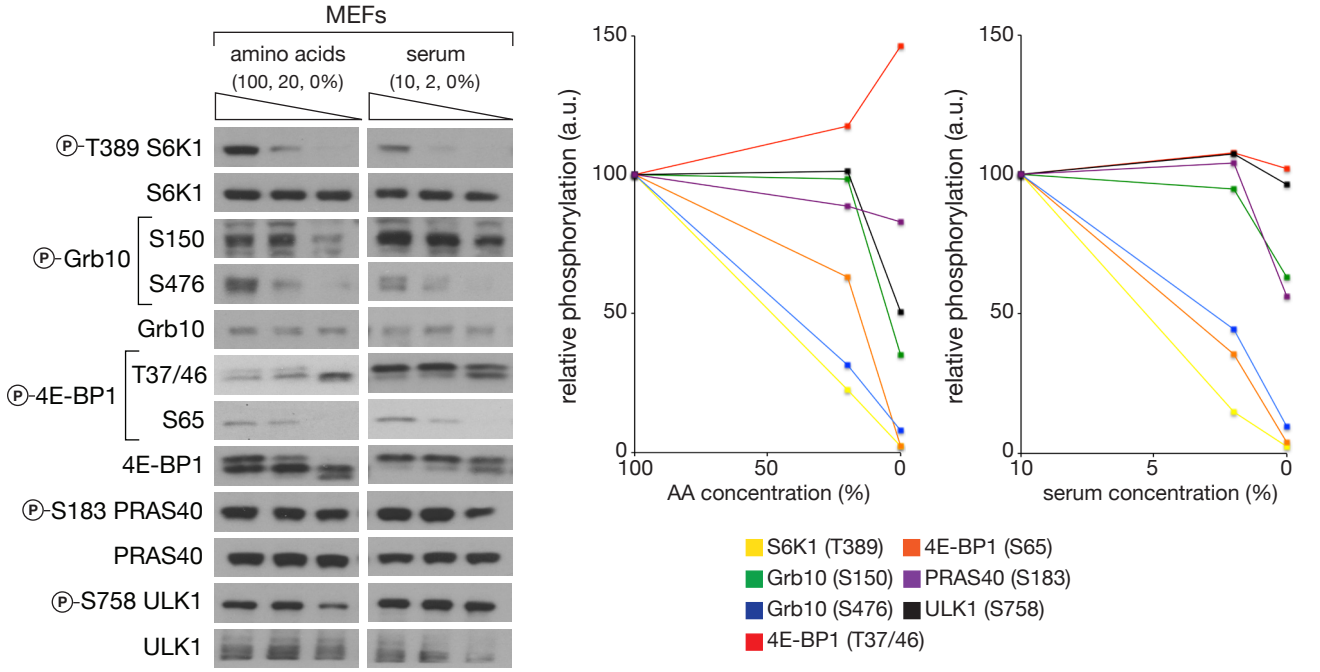
Supplemental Figure 8. Concentration-dependent responses of wild-type and mutant S758T ULK1 to rapamycin. ULK1$^{-/-}$ULK2$^{-/-}$ MEFs stably expressing FLAG-tagged wild-type or S758T ULK1 were treated with increasing concentrations of rapamycin for 2 hr in the presence of 100 nM Bafilomycin A1. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. Levels of pULK1, p62 and LC3-ii were quantified by densitometry (graphs, concentration of rapamycin in logarithmic scale) and normalized to levels of FLAG-ULK1.

Supplemental Figure 9



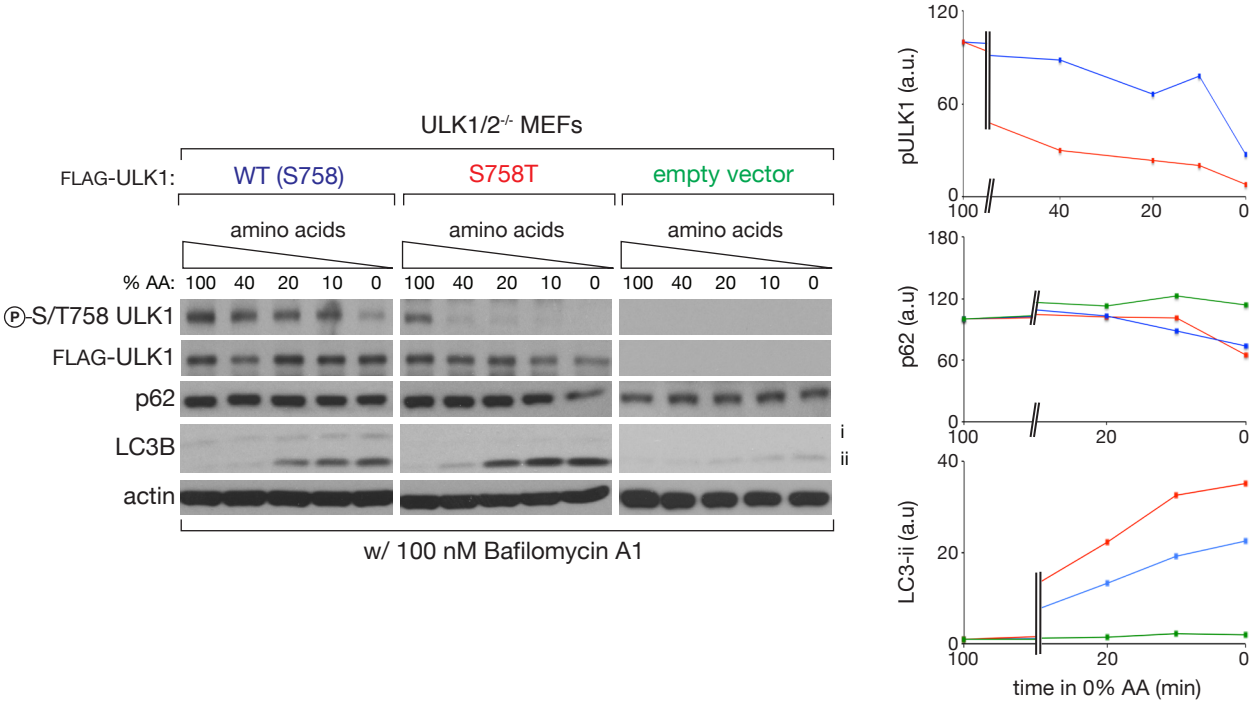
Supplemental Figure 9. A Ser-to-Thr change of the mTORC2 phosphorylation site on Akt1 is sufficient to modify its sensitivity to low dose of Torin1, but not rapamycin. HEK-293T cells were transiently transfected with GST-tagged wild-type or S473T Akt1 and treated with indicated concentrations of rapamycin and Torin1 for 60 min. Cell lysates were analyzed by immunoblotting for the level and phosphorylation state of GST-Akt1.

Supplemental Figure 10



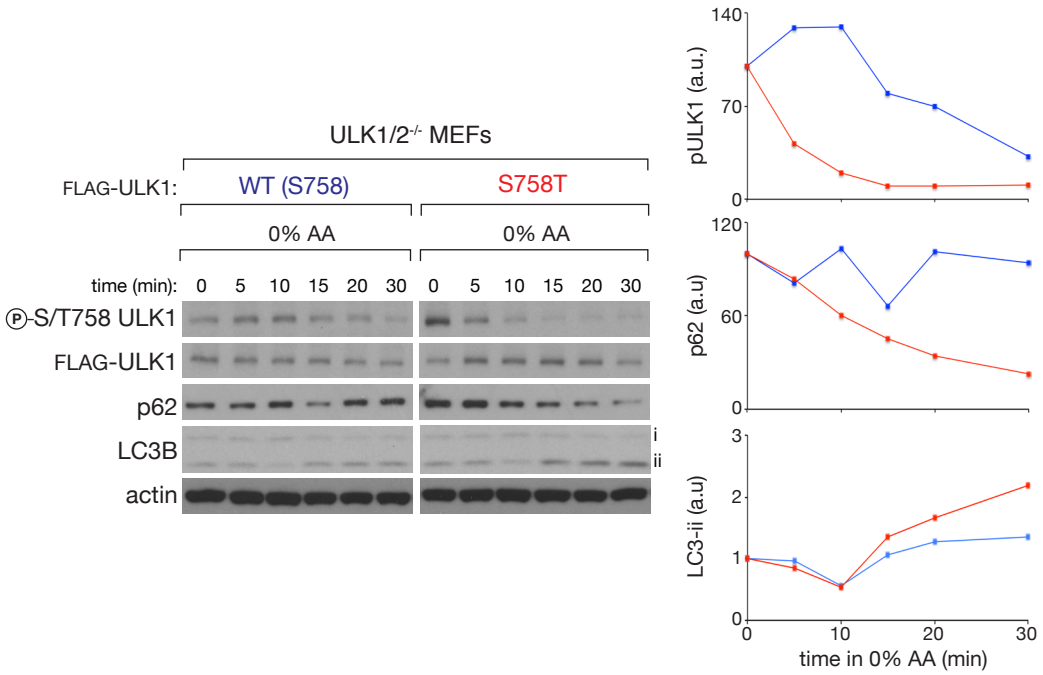
Supplemental Figure 10. Differential responses of established mTORC1 phosphorylation sites to partial amino acid or serum starvation. Quantitation by densitometry of western blots at left, which are also shown in Figure 4A. MEFs were placed in media with 100, 20 or 0% of the normal levels of amino acids or 10, 2 or 0% Fetal Bovine Serum (FBS) for 30 min. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. Phosphorylation levels of the specified proteins upon amino acid and serum starvation were quantified by densitometry (graph).

Supplemental Figure 11



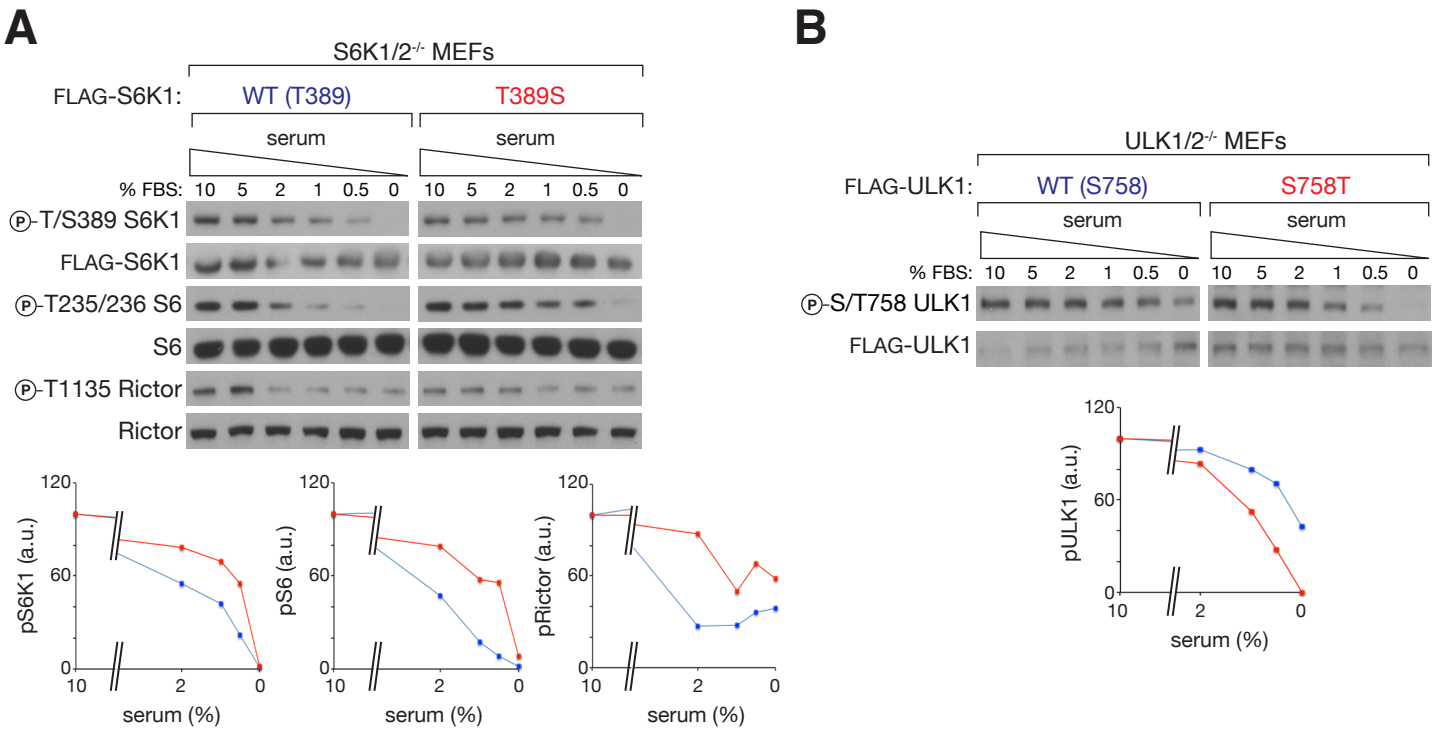
Supplemental Figure 11. Differential concentration-dependent responses of wild-type and mutant S758T ULK1 to amino acid starvation. ULK1$^{-/-}$ULK2$^{-/-}$ MEFs stably expressing FLAG-tagged wild-type ULK1, S758T ULK1 or an empty vector control were placed in media with 100, 40, 20, 10 or 0% of the normal levels of amino acids in the presence of 100 nM Bafilomycin A1 for 2 hr. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. Levels of pULK1, p62 and LC3-ii were quantified by densitometry (graphs) and normalized to levels of FLAG-ULK1 (actin for empty vector).

Supplemental Figure 12



Supplemental Figure 12. Differential time-dependent responses of wild-type and mutant S758T ULK1 to complete amino acid starvation. ULK1$^{-/-}$ULK2$^{-/-}$ MEFs stably expressing FLAG-tagged wild-type or S758T ULK1 were starved for amino acids up to 30 min. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. Levels of pULK1, p62 and LC3-ii were quantified by densitometry (graphs) and normalized to levels of FLAG-ULK1.

Supplemental Figure 13



Supplemental Figure 13. (A) Differential concentration-dependent responses of wild-type and mutant T389S S6K1 to serum starvation. S6K1$^{-/-}$S6K2$^{-/-}$ MEFs stably expressing FLAG-tagged wild-type or T389S S6K1 were placed in media with 10, 5, 2, 1, 0.5 or 0% Fetal Bovine Serum (FBS) for 20 min. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the specified proteins. Phosphorylation levels of the specified proteins upon amino acid starvation were quantified by densitometry (graphs). **(B)** Differential concentration-dependent responses of wild-type and mutant S758T ULK1 to serum starvation. Experiment was performed as in (A) with ULK1$^{-/-}$ULK2$^{-/-}$ MEFs stably expressing FLAG-tagged wild-type or S758T ULK1.